ANNOTATED SEQUENCE RECORD

Development and use of a reverse transcription insulated isothermal PCR assay for detection and characterization of bovine torovirus in yaks

Long Zhao[1] · Guoqing Shao[1] · Cheng Tang[1,2] · Hua Yue[1,2,3]

Abstract

Bovine torovirus (BToV) is an important diarrhea-causing pathogen affecting bovines. To facilitate BToV detection, a reverse transcription insulated isothermal PCR (RT-iiPCR) assay was developed that targets the BToV M gene with high specificity and reproducibility. The assay has a limit of detection of 23 copies/µL. Out of 69 diarrheic fecal samples from yaks collected on six farms in Tibet and Sichuan provinces in China, 11.59% (8/69) tested positive for BToV using this assay. The full-length spike (S) and hemagglutinin-esterase (HE) genes of three positive samples were subsequently sequenced. Notably, an identical recombination event was identified in the S1 subunit of the S protein of three isolates. All of the HE genes were found to belong to genotype III and shared the same unique aa variation (P44S) in the esterase domain. This study is the first confirmation of BToV in yaks and the first report of an S gene recombination event in BToV. Our findings will enhance the current understanding of the molecular characteristics and genetic evolution of BToV.

Introduction

Bovine torovirus (BToV) belongs to the subgenus *Renitovirus*, genus *Torovirus*, family *Tobaniviridae*, and order *Nidovirales* (ICTV: https://talk.ictvonline.org/). This diarrhea-causing bovine pathogen can also be detected in nasal samples, indicating that this virus has dual tissue tropism and implicating it as a possible causative agent for bovine respiratory disease [1–5]. To date, BToV has been reported in at least 17 countries, suggesting a worldwide geographical distribution [1, 3, 5–9].

BToVs are enveloped, single-stranded, positive-sense RNA viruses [10]. The genome contains six open reading frames (ORFs) (ORF1a, ORF1b, S, M, HE, and N) and untranslated regions (UTRs) at both ends [11]. The S, M, HE, and N ORFs encode the spike (S), membrane (M), hemagglutinin-esterase (HE), and nucleocapsid (N) protein, respectively [11]. The S protein is composed of two subunits (S1 and S2) and is involved in viral pathogenicity, host range, and tissue tropism [10, 12, 13]. The HE protein contains three functional domains: a lectin domain (R), an esterase domain (E), and a membrane-proximal domain (MP), and the R and E domains may be important for virus entry [14]. Based on their HE gene sequences, BToVs can be classified into three genotypes (I-III) [15], with genotypes II and III being the most prevalent [6]. The M protein functions in BToV assembly and nucleocapsid recognition [10]. The N protein is the only viral RNA-binding protein, and it acts in virus transcription and translation [10].

Yaks (*Bos grunniens*) belong to the genus *Bos* within the family Bovidae and are a unique long-haired bovine species. There are over 14 million yaks worldwide, distributed in high-altitude regions (above 2500–6000 m), mainly on the Qinghai–Tibet plateau in China and in parts of Nepal, India, Pakistan, Kyrgyzstan, Mongolia, and Russia [16]. Yaks are an essential source of food products such as meat and milk and also provide a means of transportation and fuel (faeces) for the local Tibetan people [17]. Diarrhea is common in newborn yaks, resulting in

Handling Editor: William G Dundon.

✉ Hua Yue
  yhua900@163.com

1   College of Animal and Veterinary Sciences, Southwest
    Minzu University, Chengdu, China

2   Key Laboratory of Veterinary Medicine of Universities
    of Sichuan Province, Chengdu, China

3   Key Laboratory of Qinghai Tibetan Plateau Animal Genetic
    Resource Reservation and Utilization, College of Animal
    and Veterinary Sciences, Southwest Minzu University,
    Chengdu 610041, China

major economic losses to the yak industry [17], and several diarrhea-causing viruses have been found circulating in yaks, including bovine rotavirus A (BRVA), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), and neboviruses (NeVs) [18–21]. However, there is currently no information regarding BToV in yaks. The goal of this study was to develop a RT-iiPCR assay to enable the rapid detection and molecular characterization of BToV in yaks.

Recently, a fluorescent probe hydrolysis-based insulated isothermal PCR (iiPCR) technology was invented and made commercially available [22]. It involves the amplification of the target cDNA by cycling the reaction components through different temperature ranges to achieve the denaturation, annealing, and extension steps of PCR [22]. Using a field-deployable PCR machine (POCKIT Nucleic Acid Analyzer, GeneRadar Biotechnology Corp., Xiamen, China), on-site detection is possible. iiPCR assays have been shown to be as sensitive as laboratory-based real-time PCR assays and have been used for the detection of many animal pathogens [22–24]. The goal of this study was to develop a RT-iiPCR assay to enable the rapid detection and molecular characterization of BToV in yaks.

Materials and methods

Viruses, bacteria, and clinical samples

Members of six virus species (bovine torovirus, bovine viral diarrhea virus, bovine rotavirus, bovine coronavirus, bovine enteritis virus, and bovine astrovirus) and four bacterial species (*Escherichia coli* K99, *Salmonella* Dublin, *Clostridium perfringens*, and *Campylobacter jejuni*) that potentially infect bovines were used to evaluate the specificity of the RT-iiPCR assay. Nucleic acid samples were preserved in our laboratory.

Forty-three diarrheic samples from dairy calves (aged ≤ 3 months) were collected from September to November 2018: 23 from Sichuan Province (Chongzhou city: 30° 39′ N 103° 40′ E, one farm) and 20 from Henan province (Zhongmou county: 34° 72′ N 113° 97′ E, one farm). These samples were used to compare detection methods.

A total of 69 diarrheic samples were collected from yaks (aged ≤ 3 months) during 2018 and 2020 (collected from June to August each year): 34 from the Tibet Autonomous Region (Ngari prefecture: 33° 42′ N 80° 11′ E, three farms) and 35 from Sichuan Province (Hongyuan county: 32° 47′ N 102° 32′ E, three farms). All samples were shipped to our laboratory on ice and stored at −80 ℃ in sterile 50-ml centrifuge tubes.

RNA extraction and cDNA synthesis

The clinical faecal samples were fully resuspended in phosphate-buffered saline (PBS) (1:5), centrifuged at 10,000 *g* for 10 min, and passed through a 0.45-μm filter. Viral RNA was extracted from 300 μl of the faecal suspension using RNAios Plus (TaKaRa Bio, Inc., Japan) according to the manufacturer's instructions. cDNA synthesis was carried out in a 20-μL reaction volume containing 4 μL of 5× Prime Script Buffer, 1 μL of Prime Script RT Enzyme Mix I, 2 μL of random hexamer primers (TaKaRa Bio, Inc.), 9 μL of RNase-free water, and 4 μL of RNA. The mixtures were incubated at 37 °C for 15 min, 85 °C for 15 s, and 16 °C for 10 min and then stored at -20 °C.

RT-iiPCR

Primer design

The primers and probe were designed based on the complete M gene sequences (GenBank database) of all 13 BToV strains. A 114-bp segment of the M gene sequence was chosen as the target using Beacon Designer 8. The primer sequences were as follows: F, 5'-GCTTGTTGCACTTGA TRTTA-3; R, 5'-GCTGTTAATGGTTTATTGC-3'; and the TaqMan probe sequence was FAM-5'-AAGATGTTGCCA ACCTCCTTAACAA-3'-BHQ1.

Optimization of RT-iiPCR

Various concentrations of components were tested and screened in a total volume of 50 μL, which included the 10 μM forward and reverse primers (0.5-4 μL), 10 μM probe (0.05-0.4 μL), 16.6-23.95 μL of RNase-free water, and 25 μL of Premix Ex Taq DNA polymerase (5 U/μL) (TaKaRa Biotechnology, Dalian, China). The RT-iiPCR was carried out using a POCKIT™ device (GeneRadar Biotechnology Corp), with default parameters and an iiPCR step at 95℃ for 58 minutes. Optimum conditions were determined by measuring the ratio between the final and initial fluorescence intensities (A502/B520).

Specificity of RT-iiPCR

Ten major bovine diarrhea-causing pathogens (listed above) were used to determine the specificity of the RT-iiPCR.

Sensitivity of RT-iiPCR

A BToV standard plasmid was prepared as described by Hosmillo et al. [25]. Briefly, the targeted 603-bp M gene

fragment was cloned into the pMD19 Simple T Vector (TaKaRa Bio Inc.) and introduced into competent *Escherichia coli* DH5α cells (Yeasen). The plasmids were extracted using a MiniBEST Plasmid Purification Kit (TaKaRa Bio Inc.) and sequenced (Sangon Biotech) in both directions. The copy number was determined by SYBR Green real-time RT-PCR [25]. Tenfold serial dilutions of the standard plasmid were prepared in RNase-free water and assayed by RT-iiPCR to evaluate the sensitivity of the test. All samples were amplified under the optimum conditions.

Reproducibility of RT-iiPCR

Six different dilutions of the standard plasmid (10^{-4}-10^{-9}) were used to evaluate the reproducibility of the RT-iiPCR. Each sample was amplified in triplicate.

Comparison with SYBR Green real-time RT-PCR

A total of 43 clinical diarrhea samples collected from dairy calves were analyzed simultaneously by SYBR Green real-time RT-PCR and RT-iiPCR, and the agreement between the two methods was calculated. Each sample was amplified in triplicate.

Screening for BToV by RT-iiPCR

A total of 69 clinical samples (described above) were tested for the presence of BToV using the RT-iiPCR assay established in this study.

Amplification of complete S and HE gene sequences

BToV-positive samples were used to simultaneously amplify the complete S and HE genes. The primer information is presented elsewhere [6]. All amplification products were purified and cloned into PMD19 Simple T Vector (TaKaRa Bio, Inc.) for sequencing, and the resulting sequences were assembled using SeqMan software (version 7.0; DNASTAR, Madison, WI, USA).

Sequence, phylogeny, recombination, and structural analysis

MEGA 7.0.26 was used to perform multiple sequence alignment using ClustalW and subsequently to build a phylogenetic tree, which was constructed by the maximum-likelihood method with bootstrap values calculated for 1000 replicates, Poisson correction, and an amino acid substitution model [26]. Nucleotide and deduced amino acid sequence identity values were determined using the MegAlign program in DNASTAR 7.0 (DNASTAR, Inc.). Recombination events were assessed using SimPlot

software (version 3.5.1) and RDP 4.97, using the RDP, GeneConv, Chimaera, MaxChi, BootScan, SiScan, 3Seq, and Phylpro methods [27]. 3D models were constructed based on the crystal structure of bovine coronavirus hemagglutinin-esterase (SMTL ID: 3i26.1) using the online software SWISSMODEL (https://www.swissmodel.expasy.org/interactive) [14].

Results

RT-iiPCR

The optimal reaction volume was determined based on the maximum A520/B520 value of 2.5 using 4 µL of each primer (10 µM), 0.35 µL of probe (10 µM), 16.65 µL of nuclease-free water, and 25 µL of Premix Ex Taq DNA polymerase (5 U/µL) in a total volume of 50 µL. In this reaction volume, BToV could be detected by RT-iiPCR, and there was no amplification of the other pathogens tested. The limit of detection was 23 copies/µL, indicating the high sensitivity of this RT-iiPCR assay for the detection of BToV. (Note: the sensitivity was determined using standard positive plasmids without the background genetic material that would be present in a clinical sample.) To assess the repeatability of the assay, six samples of standard plasmid were assayed in triplicate with tenfold serial dilutions (from 10^{-4} to 10^{-9}), and all samples were positive (Table 1). The consistency of this analysis is indicative of the high reproducibility of the RT-iiPCR.

Forty-three previously collected diarrhea samples were selected to compare the RT-iiPCR and SYBR Green real-time RT-PCR methods. Both assays gave identical results for each sample (Table 2), with a 41.86% (18/43) positive rate, indicating a high level of agreement between the RT-iiPCR and SYBR Green real-time RT-PCR systems for detection of BToV.

Table 1 RT-iiPCR repeatability

Plasmid dilution	Positive (runs 1-3)	Average A520/B520 ± SD	%CV
No template	0/3	0.99 ± 0.014	1.41
10^{-4}	3/3	2.62 ± 0.001	0.04
10^{-5}	3/3	2.56 ± 0.012	0.47
10^{-6}	3/3	2.52 ± 0.012	0.48
10^{-7}	3/3	2.19 ± 0.047	2.15
10^{-8}	3/3	1.92 ± 0.037	1.93
10^{-9}	3/3	1.64 ± 0.036	2.20

Table 2 Comparison of BToV RT-iiPCR and SYBR Green real-time RT-PCR

Sample ID	RT-iiPCR (Average A520/B520 ± SD)	SYBR Green real-time RT-PCR (average Ct value ± SD)
SC01	+ (1.98 ± 0.082)	32.58 ± 0.187
SC01	+ (1.70 ± 0.050)	34.36 ± 0.328
SC03	+ (1.76 ± 0.111)	33.18 ± 0.288
SC04	+ (1.99 ± 0.096)	34.64 ± 0.421
SC05	+ (2.1 ± 0.155)	33.36 ± 0.469
SC06	-	-
SC07	-	-
SC08	-	-
SC09	-	-
SC10	+ (2.21 ± 0.092)	31.74 ± 0.462
SC11	+ (1.81 ± 0.062)	33.99 ± 0.130
SC12	+ (2.11 ± 0.142)	33.59 ± 0.072
SC13	+ (2.76 ± 0.236)	14.34 ± 0.329
SC14	+ (2.06 ± 0.066)	32.98 ± 0.101
SC15	-	-
SC16	-	-
SC17	+ (2.56 ± 0.050)	26.79 ± 0.353
SC18	+ (2.26 ± 0.089)	26.73 ± 0.524
SC19	+ (1.96 ± 0.111)	33.42 ± 0.260
SC20	+ (2.36 ± 0.210)	20.88 ± 0.325
SC21	+ (2.16 ± 0.046)	33.12 ± 0.570
SC22	-	-
SC23	+ (2.66 ± 0.017)	19.90 ± 0.275
LN01	+ (2.16 ± 0.020)	31.26 ± 0.320
LN02	+ (2.09 ± 0.026)	32.35 ± 0.922
LN03	-	-
LN04	-	-
LN05	-	-
LN06	-	-
LN07	-	-
LN08	-	-
LN09	-	-
LN10	-	-
LN11	-	-
LN12	-	-
LN13	-	-
LN14	-	-
LN15	-	-
LN16	-	-
LN17	-	-
LN18	-	-
LN19	-	-
LN20	-	-

−, negative; +, positive

Detection of BToV in yaks

Of the 69 diarrheic samples tested, 11.59% (8/69) were BToV positive by RT-iiPCR. Notably, BToV was found in samples collected from four farms in two provinces, with positive rates of 11.76% (4/34) and 11.43% (4/35) for samples collected in the Tibet Autonomous Region and Sichuan Province, respectively.

Molecular characterization of the S gene

Full-length S genes were successfully cloned from three positive samples from two different farms, one in the Tibet Autonomous Region and one in Sichuan Province, more than 1500 km apart (GenBank accession numbers MW114530, MW114531, and MN882587.1). The three S genes were each 4755 bp in length, encoding a protein of 1584 amino acids (aa). These three S genes shared 99.3%-99.9% nt and 99.2%-99.9% aa sequence identity with each other, and 91.5%-99.6% nt and 89.8%-99.6% aa sequence identity with all 22 full-length BToV S gene sequences available in the GenBank database.

A phylogenetic tree was constructed by the maximum-likelihood method based on the complete amino acid sequence of the S protein, and this showed that the three yak BToV strains were most closely related to Chinese dairy cow strains (GenBank accession nos. MT364039, MT364036, MT364037, and MT364041). Two strains from the Tibet Autonomous Region clustered separately in a small branch (Fig. 1). Compared to the 22 available full-length BToV S gene sequences, the strains from the Tibet Autonomous Region, XZ01 and XZ02, contained three amino acid mutations (S35F, T1335I, and K1583R) (Fig. S1-S3). One of these substitutions (S35F) was located in the S1 subunit, and the others were in the S2 subunit.

Next, RDP 4.97 and SimPlot 3.5.1 were used to perform recombination analysis of the 34 available full-length ToV S gene sequences (25 BToV strains, six PToV strains, one EToV strain, one goat strain, and one *Sarcophilus harrisii* strain). Interestingly, recombination events had occurred in all three yak BToV strains. The recombination breakpoint was determined by analysis using RDP 4.97 (six methods: RDP, GeneConv, Chimaera, MaxChi, SiScan, and 3Seq; recombinant score: 0.581) as beginning at nt 220 in the fragment (breakpoint 99% confidence intervals: nt position 1-285 in the fragment) and ending at nt 2296 (breakpoint 99% confidence intervals: nt position 2198-2370 in the fragment) in the putative major parental strain HT2 (GenBank accession no. MG957146.1) and the possible minor parental strain Breda 1 (GenBank accession no. AY427798.1) (Fig. 2). Moreover, using SimPlot 3.5.1, a standard similarity plot analysis showed that the recombination region was mapped to nt 224–2279. Although the positions of

Fig. 1 Phylogenetic tree based on the complete deduced 1584-aa sequence of the S protein. Sequence alignments and clustering were performed using ClustalW in MEGA 7.0 software. The tree was constructed by the maximum-likelihood method with bootstrap values calculated for 1,000 replicates. The isolates from this study are indicated by a triangle, and the other Chinese BToV isolates are indicated by a square.
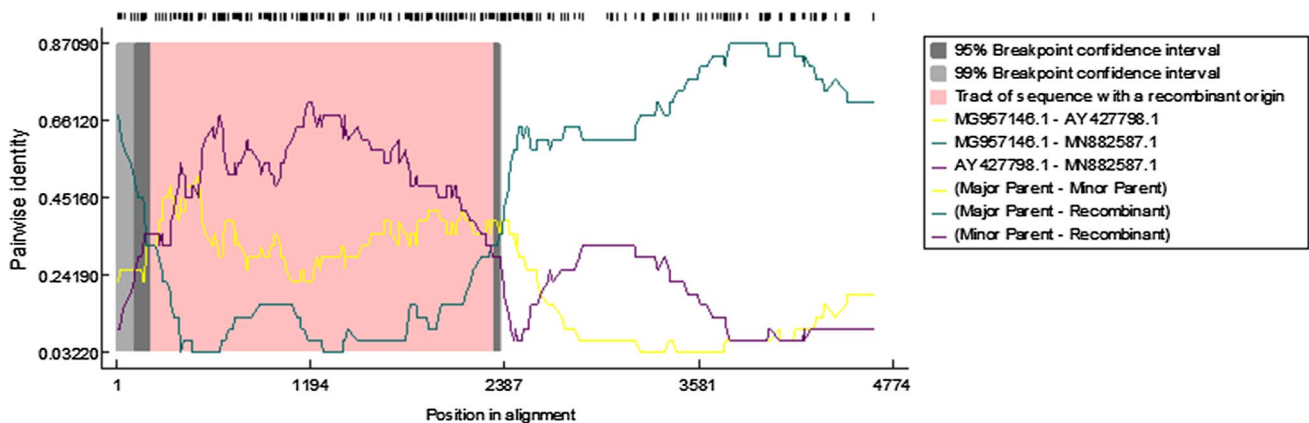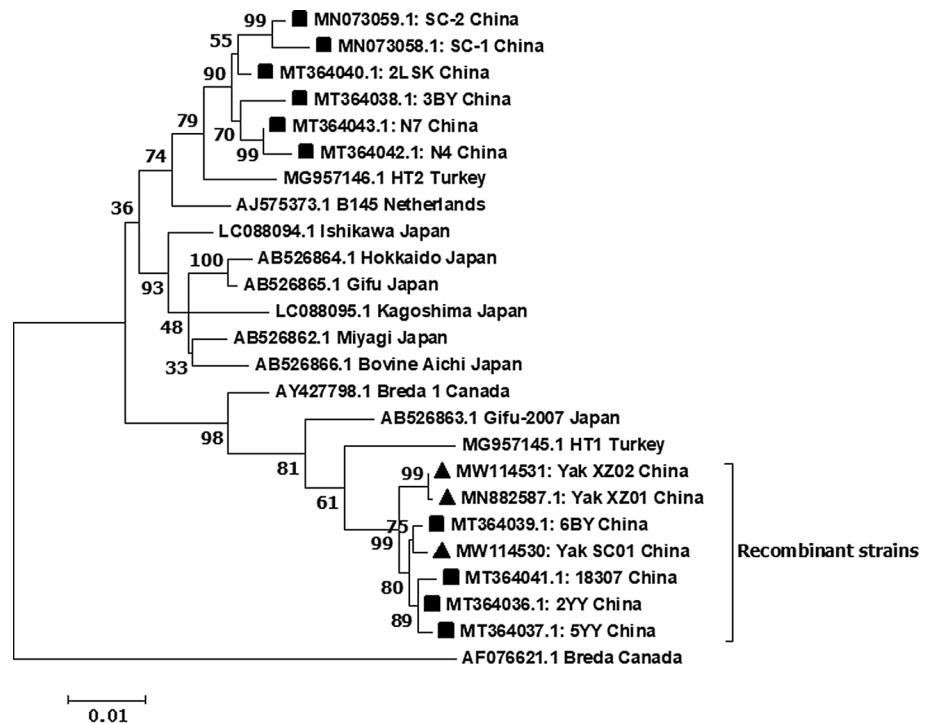
Fig. 2 Recombination analysis of strain XZ01 using RDP 4.97. A nucleotide (nt) sequence identity plot comparing the S gene (4755 nt) of strain XZ01 (MN882587.1) with those of the BToV strains HT2 (GenBank accession no. MG957146.1) and Breda 1 (GenBank accession no. AY427798.1) is shown. The recombination breakpoint was identified using RDP 4.97 as beginning at nt 220 in the fragment (breakpoint 99% confidence intervals: nt position 1-285 in the gray fragment) and ending at nt 2296 (breakpoint 99% confidence inter-vals: nt position 2198-2370 in the gray fragment) in the putative major parental strain HT2 (GenBank accession no. MG957146.1) and the possible minor parental strain Breda 1 (GenBank accession no. AY427798.1). The putative recombination region is located at nt 220-2296 (shown in pink). The vertical axis indicates the identity (%) of nucleotide sequences between the query strain and other reference strains. The horizontal axis indicates the nucleotide position. (Only one recombinant strain is shown due to space limitations.)

the recombination breakpoints predicted by RDP 4.97 and SimPlot differed somewhat, both programs showed that the recombination breakpoint was located in the S1 subunit. The phylogenetic trees also provide supporting evidence of recombination events the S gene. Furthermore, four Chinese strains isolated from cattle (GenBank accession nos. MT364039, MT364036, MT364037, and MT364041) that clustered with the three recombinant strains in the phylogenetic tree (Fig. 1) were also identified as recombinant strains with the same recombination point, indicating the same recombination event.

Molecular characterization of the HE gene

The full-length HE genes were successfully sequenced from the three positive samples that were used to amplify the S

gene fragments (GenBank accession nos. MW114528, and MW114529, MN882587.1). All three HE genes were 1,257 bp long, encoding a protein 418 aa residues in length. The HE gene sequences shared 98.7%-99.8% nt and 99.0%-99.8% aa sequence identity with each other and shared 71.7%-99.1% nt and 71%-98.8% aa sequence identity with all 24 of the BToV HE genes with complete sequences available in the GenBank database.

A phylogenetic tree was constructed by the maximum-likelihood method based on the complete aa sequence of the HE protein, and this showed that all three yak BToV strains detected in this study were most closely related to a Chinese cattle strain previously obtained in our laboratory (GenBank accession no. MW114525) that belongs to genotype III (Fig. 3). Compared to the four available genotype III sequences, all three yak BToV genotype III strains had a single amino acid mutation (P44S) (Fig. S4), located in the esterase domain. In addition, compared with the sequences of the three foreign genotype III strains in the GenBank database, there were five amino acid mutations in the HE gene of the Chinese genotype III strains (F6L, V78A, E143A, S238T, and Y418H) (Fig. S4).

A 3D model was constructed, and the results showed a significant difference in the three yak strains compared to the other four genotype III strains in GenBank (including one Chinese cow strain, two strains from the Netherlands, and one strain from Turkey). The three yak genotype III strains are predicted to have an α-helix from amino acids 238 to 240 in the lectin domain, whereas the other type III strains are predicted to have a random coil in this region (Fig. 4).
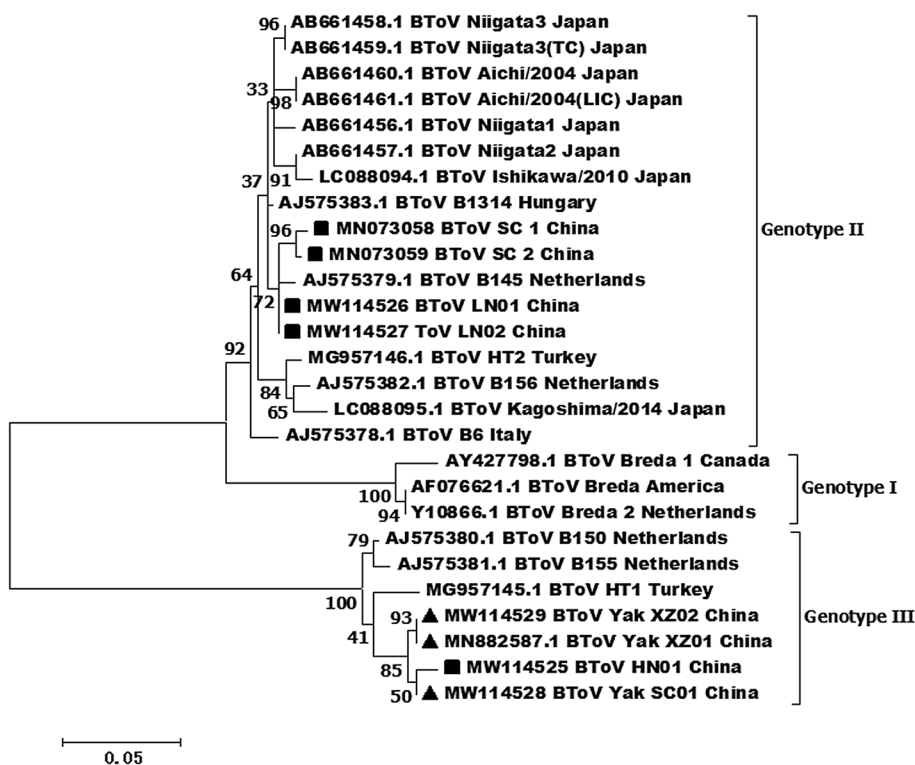
Discussion

Development of RT-iiPCR assay

BToV is an important diarrhea-causing pathogen that has been detected in China, Japan, Turkey, Italy, Brazil, America, South Africa, and other countries [1, 3, 5, 6, 8, 15, 28]. In this study, we developed a new RT-iiPCR assay for the detection of BToV based on the portable, commercially available POCKIT™ device. This assay demonstrated high specificity and reproducibility for detection of BToV RNA with a detection limit of 23 copies/μL, thus providing an effective new tool for BToV detection. The RT-iiPCR assay takes only 58 minutes to produce a result, a much shorter time than with other PCR methods. POCKIT™ devices for RT-iiPCR are widely used for detection of pathogens in the field [23, 29, 30]. Therefore, rapid detection of BToV can be carried out in the field by RT-iiPCR using a field nucleic acid extraction kit.

Detection of BToV in yaks

The Qinghai-Tibet Plateau is 4000 m above sea level on average, with strong ultraviolet radiation and an annual

Fig. 3 Phylogenetic tree based on the complete deduced 418-aa sequence of the HE protein. Sequence alignments and clustering were performed using ClustalW in MEGA 7.0 software. The tree was constructed by the maximum-likelihood method with bootstrap values calculated for 1,000 replicates. The isolates from this study are indicated by a triangle, and the other Chinese BToV isolates are indicated by a square.
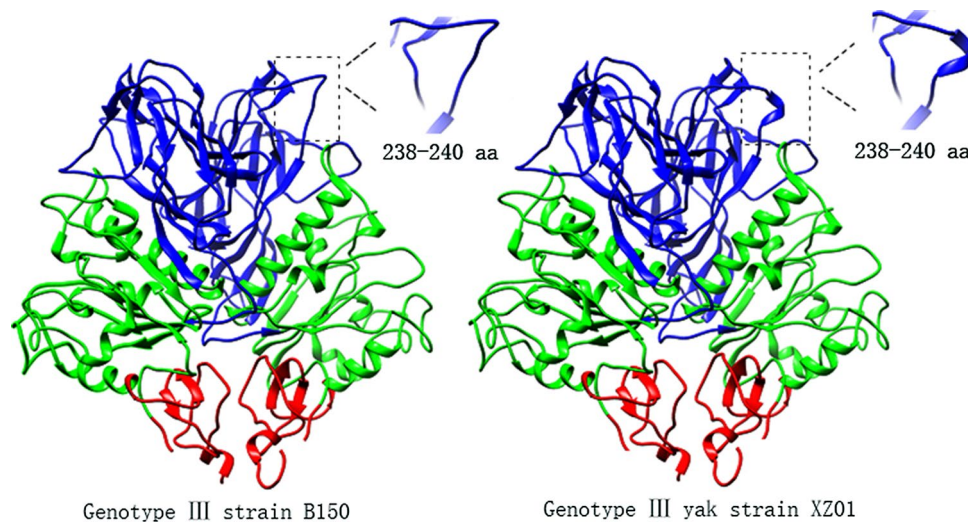
Genotype III strain B150   Genotype III yak strain XZ01

Fig. 4 Predicted 3D structures of the BToV HE proteins (aa residues 15-392) of BToV strain B150 (GenBank accession no. AJ575380.1) and yak BToV strain XZ01 (GenBank accession no. MN882587.1). The 3D models were constructed based on the crystal structure of BToV hemagglutinin-esterase (SMTL ID: 3i26.1) using the online software SWISSMODEL (https://swissmodel.expasy.org/interactive). These models have identical structures in the esterase domain and the membrane-proximal domain. Yak BToV genotype III strains differ from the other genotype III strains in the lectin domain. The boxed portions of the structures indicate the different conformations of the same R domain of BToV strains. The domains are color-coded: lectin domain (R, blue); esterase domain (E, green); membrane-proximal domain (MP, red).

average temperature of less than 10 ℃ in most areas [17]. Yaks are essential for the livelihood of the local Tibetan people, providing meat, milk, skins, transport, and fuel (faeces) [17]. In this study, 11.59% of the samples collected from yaks were BToV positive by RT-iiPCR, and the virus was detected on four out of the six farms tested in the two provinces in China. Although this is a low positivity rate for BToV, this is the first confirmation of the presence of BToV in yaks. BToV may already have a wide geographical distribution, and future work should determine the prevalence of BToV in yaks. In addition to China, yaks are distributed in India, Nepal, Pakistan, Kyrgyzstan, Mongolia, and Russia [16]. Diarrhea in newborn yaks causes major economic losses in the yak industry [31], and the results of this study provide a reference for the diagnosis and control of diarrhea in yaks. Phylogenetic analysis based on the S and HE genes showed that the yak BToV strain is closely related to cattle BToV strains from China, suggesting that the yak strain may have been transmitted from Chinese cattle.

Molecular characterization of the S gene

The S protein of BToV, like its counterpart in BCoV, is involved in the induction of neutralizing antibody production during the infection process and plays an important role in pathogenesis [10, 32–34]. Cleavage occurs between amino acids 1003 and 1007 of the mature S protein of BToV, resulting in the formation of the S1 and S2 subunits [34, 35]. S1 can bind independently to cellular receptors, and S2 mediates fusion of the viral and cell membranes [32]. The two yak BToV isolates from Tibet in this study have three identical amino acid mutations (S35F, T1335I, and K1583R). The first amino acid mutation is located in the S1 subunit, and the other two are located in the S2 subunit. These amino acid mutations might affect the function of the S protein. Further work is needed to investigate the biological significance of these amino acid mutations. Recombination facilitates RNA virus evolution and can result in the emergence of new pathotypes, as well as the expansion of host ranges and ecological niches [36, 37]. Recombination occurring in the S and HE genes has been reported extensively for coronaviruses, and recombination has also been reported in the HE gene in BToV [15, 38, 39]. In our study, all three yak BToV isolates appeared to have undergone the same recombination event in the S1 subunit of the S protein. Since the S1 subunit binds the host receptor [32], these sequence changes may have biological consequences and should be studied further. Due to the small number of sequences obtained in this study, the prevalence of this recombinant strain needs further investigation.

Molecular characterization of the HE gene

To date, there are only 27 complete BToV HE gene sequences in the GenBank database (including the sequences obtained in this study). Analysis of these sequences has shown that there are three genotypes of BToV [15]. Sequences are available for three genotype I isolates, 17

genotype II isolates, and seven genotype III isolates (including the three sequences obtained in this study). Genotype III strains have only been reported in China (GenBank accession no. MW114525), Turkey (GenBank accession no. MG957145.1), and the Netherlands [15]. Interestingly, compared with the other four genotype III strains, the three yak BToV isolates from this study shared an identical unique amino acid change (P44S) in the E region. The esterase domain possesses receptor-destroying activity, which enables the virus to separate from sialic acid on the cell surface [14], and this sequence variation may affect the esterase function of the HE protein. This unique amino acid change in the yak BToV strain might be a specific adaptation to yaks or be related to the special geographical environment of the Qinghai-Tibet Plateau, with high altitude (average altitude of over 4000 m), low oxygen, low temperature, and low atmospheric pressure.

A 3D model showed that the HE proteins of the three yak genotype III isolates were predicted to contain an α-helix from amino acids 238 to 240 in the lectin domain, while the other genotype III sequences were predicted to contain a random coil in this part of the protein [14]. Interestingly, compared with the other four genotype III sequences in GenBank, the three yak genotype III strains showed no unique amino acid variations at positions 238-240. The significance of this conformational difference in this portion of the protein should be studied further.

In conclusion, an RT-iiPCR assay was developed that provides a new tool for the rapid detection of BToV. This assay was used to identify BToV for the first time in yaks. A novel S gene recombination event was identified in BToV, and a unique amino acid change (P44S) was detected in the E region of the HE protein, which might affect esterase function. These findings will enhance our understanding of the epidemic and the genetic evolution of BToV.

Data availability statement

The GenBank accession numbers of sequences described in this article are as follows: AY427798.1, AF076621.1, Y10866.1, MG957146.1, AJ575383.1, AJ575382.1, AJ575379.1, AJ575378.1, AB661458.1, AB661459.1, AB661457.1, AB661456.1, AB661460.1, AB661461.1, LC088095.1, LC088094.1, AJ575380.1, AJ575381.1, MG957145.1, MW114525, MW114526, MW114527, MW114529, MW114528, AJ575373.1, AB526862.1, AB526864.1, AB526865.1, AB526863.1, AB526866.1, MT364039.1, MT364036.1, MT364037.1, MT364041.1, MT364040.1, MT364043.1, MN073059.1, MT364042.1, MN073058.1, MT364038.1, MN882587.1, MW114531, MW114530, JQ860350.1, GU196786.1, MH603532.1, LC483442.1, KM403390.1, AJ575372.1, MK521914.1, MG996765.1, KR527150.1.

Declarations

Conflict of interest All authors report no conflict of interest related to the submitted work.

Ethical approval This study did not involve animal experiments other than analysis of fecal samples of diarrheic yak calves collected during visits to farms for clinical treatment.

References

1. Hoet A, Cho K, Chang K, Loerch S, Wittum T, Saif L (2002) Enteric and nasal shedding of bovine torovirus (Breda virus) in feedlot cattle. Am J Vet Res 63(3):342–348. https://doi.org/10.2460/ajvr.2002.63.342

2. Hoet A, Smiley J, Thomas C, Nielsen P, Wittum T, Saif L (2003) Association of enteric shedding of bovine torovirus (Breda virus) and other enteropathogens with diarrhea in veal calves. Am J Vet Res 64(4):485–490. https://doi.org/10.2460/ajvr.2003.64.485

3. Ito T, Okada N, Okawa M, Fukuyama SI, Shimizu M (2009) Detection and characterization of bovine torovirus from the respiratory tract in Japanese cattle. Vet Microbiol 136(3–4):366–371. https://doi.org/10.1016/j.vetmic.2008.11.014

4. Aita T, Kuwabara M, Murayama K, Sasagawa Y, Yabe S, Higuchi R, Tamura T, Miyazaki A, Tsunemitsu H (2012) Characterization of epidemic diarrhea outbreaks associated with bovine torovirus in adult cows. Adv Virol 157(3):423–431. https://doi.org/10.1007/s00705-011-1183-9

5. Nogueira JS, Asano KM, De Souza SP, Brandão PE, Richtzenhain LJ (2013) First detection and molecular diversity of Brazilian bovine torovirus (BToV) strains from young and adult cattle. Res Vet Sci 95(2):799–801. https://doi.org/10.1016/j.rvsc.2013.04.006

6. Li H, Zhang B, Yue H, Tang C (2020) First detection and genomic characteristics of bovine torovirus in dairy calves in China. Arch Virol 165(4):1577–1583. https://doi.org/10.1007/s00705-020-04657-9

7. I L, N K, I Š, T B, (2015) Detection and molecular characterisation of bovine corona and toroviruses from Croatian cattle. BMC Vet Res 11:202. https://doi.org/10.1186/s12917-015-0511-9

8. Gülaçtı I, Işıdan H, Sözdutmaz I (2014) Detection of bovine torovirus in fecal specimens from calves with diarrhea in Turkey. Adv Virol 159(7):1623–1627. https://doi.org/10.1007/s00705-014-1977-7

9. Woode G, Reed D, Runnels P, Herrig M, Hill H (1982) Studies with an unclassified virus isolated from diarrheic calves. Vet Microbiol 7(3):221–240. https://doi.org/10.1016/0378-1135(82)90036-0

10. Hoet A, Saif L (2004) Bovine torovirus (Breda virus) revisited. Anim Health Res Rev 5(2):157–171. https://doi.org/10.1079/ahr200498

11. Draker R, Roper R, Petric M, Tellier R (2006) The complete sequence of the bovine torovirus genome. Virus Res 115(1):56–68. https://doi.org/10.1016/j.virusres.2005.07.005

12. Peng G, Xu L, Lin YL, Chen L, Pasquarella JR, Holmes KV, Li F (2012) Crystal structure of bovine coronavirus spike protein lectin domain. J Biol Chem 287(50):41931. https://doi.org/10.1074/jbc.M112.418210

13. Gallagher TM, Buchmeier MJ (2001) Coronavirus spike proteins in viral entry and pathogenesis. Virology 279(2):371–374. https://doi.org/10.1006/viro.2000.0757

14. Langereis MA, Zeng Q, Gerwig GJ, Frey B, Itzstein MV, Kamerling JP, De Groot RJ, Huizinga EG (2009) Structural basis for ligand and substrate recognition by torovirus hemagglutinin esterases. Proc Natl Acad USA Huizinga 106(37):15897–15902. https://doi.org/10.1073/pnas.0904266106

15. Smits SL, Lavazza A, Matiz K, Horzinek MC, Koopmans MP, De Groot RJ (2003) Phylogenetic and evolutionary relationship among torovirus field variants: evidence for multiple intertypic recombination events. J Virol 77(17):9567–9577. https://doi.org/10.1128/JVI.77.17.9567-9577.2003

16. Liu J, Cai J, Zhang W, Liu Q, Chen D, Han J, Liu Q (2008) Seroepidemiology of Neospora caninum and Toxoplasma gondii infection in yaks (*Bos grunniens*) in Qinghai, China. Vet Parasitol 152:330–332. https://doi.org/10.1016/j.vetpar.2007.12.010

17. Guo X, Long R, Kreuzer M, Ding L, Shang Z, Zhang Y, Yang Y, Cui G (2014) Importance of functional ingredients in yak milk-derived food on health of Tibetan nomads living under high-altitude stress: a review. Crit Rev Food Sci Cui Nutr 54(3):292–302. https://doi.org/10.1080/10408398.2011.584134

18. Yan N, Li R, Wang Y, Zhang B, Tang C (2020) High prevalence and genomic characteristics of G6P[1] Bovine Rotavirus A in yak in China. J Gen Virol 101(7):701–711. https://doi.org/10.1099/jgv.0.001426

19. He Q, Guo Z, Zhang B, Yue H, Tang C (2019) First detection of bovine coronavirus in Yak (*Bos grunniens*) and a bovine coronavirus genome with a recombinant HE gene. J Gen Virol 100(5):793. https://doi.org/10.1099/jgv.0.001254

20. Guo Z, He Q, Zhang B, Yue H, Tang C (2019) First detection of neboviruses in Yak (*Bos grunniens*) and identification of a novel nebovirus based on complete genome. Vet Microbiol 236:108388

21. Gong X, Liu L, Zheng F, Chen Q, Li Z, Cao X, Yin H, Zhou J, Cai X (2014) Molecular investigation of bovine viral diarrhea virus infection in yaks (*Bos gruniens*) from Qinghai, China. Virol J 11(1):1–7. https://doi.org/10.1186/1743-422X-11-29

22. Tsai YL, Wang HC, Lo CF, Tang-Nelson K, Lightner D, Ou BR, Hour AL, Tsai CF, Yen CC, Chang HF, Teng PH, Lee PY (2014) Validation of a commercial insulated isothermal PCR-based POCKIT test for rapid and easy detection of white spot syndrome virus infection in *Litopenaeus vannamei*. PLoS ONE 9(3):e90545. https://doi.org/10.1371/journal.pone.0090545

23. Ambagala A, Fisher M, Goolia M, Nfon C, Furukawa-Stoffer T, Ortega Polo R, Lung O (2017) Field-deployable reverse transcription-insulated isothermal PCR (RT-iiPCR) Assay for rapid and sensitive detection of foot-and-mouth disease virus. Transbound Emerg Dis 64(5):1610–1623. https://doi.org/10.1111/tbed.12554

24. Ambagala A, Pahari S, Fisher M, Lee PYA, Pasick J, Ostlund EN, Johnson DJ, Lung O (2015) A rapid field-deployable reverse transcription-insulated isothermal polymerase chain reaction assay for sensitive and specific detection of bluetongue virus. Transbound Emerg Dis 64:2. https://doi.org/10.1111/tbed.12388

25. Hosmillo M, Jeong Y, Kim H, Collantes T, Alfajaro M, Park J, Kim H, Kwon H, Park S, Kang M, Park S, Cho KO (2010) Development of universal SYBR Green real-time RT-PCR for the rapid detection and quantitation of bovine and porcine toroviruses. J Virol Methods 168:212–217. https://doi.org/10.1016/j.jviromet.2010.06.001

26. Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5(2):150–163. https://doi.org/10.1093/bib/5.2.150

27. Martin DP, Ben M, Michael G, Arjun K, Brejnev M (2015) RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol 1(1):003. https://doi.org/10.1093/ve/vev003

28. Vj H, Gg H (1993) Breda virus-like particles in calves in South Africa. J S Afr Vet Assoc Gerdes 64(2):58

29. Chua KH, Lee PC, Chai HC (2016) Development of insulated isothermal PCR for rapid on-site malaria detection. Malar J 15(1):134. https://doi.org/10.1186/s12936-016-1183-z

30. Lee TPY (2012) Detection of white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions. J Virol Methods 181(1):134–137. https://doi.org/10.1016/j.jviromet.2012.01.017

31. Chen X, Zhang B, Yue H, Wang Y, Zhou F, Zhang Q, Tang C (2015) A novel astrovirus species in the gut of yaks with diarrhoea in the Qinghai-Tibetan Plateau, 2013. J Gen Virol 96(12):3672–3680. https://doi.org/10.1099/jgv.0.000303

32. Li F (2016) Structure, function, and evolution of coronavirus spike proteins. Annu Rev Virol 3(1):1954–1964. https://doi.org/10.1146/annurev-virology-110615-042301

33. Koopmans M, Ederveen J, Woode GN, Horzinek MC (1986) Surface proteins of Breda virus. Am J Vet Res 47(9):1896

34. Zhang-Min Hu, Yong-Le Y, Ling-Dong Xu, Bin W, Pan Q (2019) Porcine Torovirus (PToV)-a brief review of etiology, diagnostic assays and current epidemiology. Front Vet Sci 6:120. https://doi.org/10.3389/fvets.2019.00120

35. Lynn D, Raymond T, Peter L (1998) Bovine torovirus: Sequencing of the structural genes and expression of the nucleocapsid protein of Breda virus. Virus Res 58:83–96. https://doi.org/10.1016/s0168-1702(98)00104-x

36. Li F (2013) Receptor recognition and cross-species infections of SARS coronavirus. Antiviral Res 100(1):246–254. https://doi.org/10.1016/j.antiviral.2013.08.014

37. Graham RL, Baric RS (2009) Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J Virol 84(7):3134–3146. https://doi.org/10.1128/JVI.01394-09

38. Keha A, Xue L, Yan S, Yue H, Tang C, Diseases E (2019) Prevalence of a novel bovine coronavirus strain with a recombinant hemagglutinin/esterase gene in dairy calves in China. Transbound Emerg Dis 66(5):1971–1981. https://doi.org/10.1111/tbed.13228

39. Minami S, Kuroda Y, Terada Y, Yonemitsu K, Van Nguyen D, Kuwata R, Shimoda H, Takano Ai, Maeda K (2016) Detection of novel ferret coronaviruses and evidence of recombination among ferret coronaviruses. Virus Genes 52(6):858–862. https://doi.org/10.1007/s11262-016-1365-3